SEROLOGICAL METHODS IN THE STUDY OF TRANSPLANTATION ANTIGENS

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WHEN lymphoid cells or other grafts from A-strain mice are transplanted into mice of strains CBA or C3H, they produce effects of 2 distinct kinds : (a) they excite transplantation immunity, as shown by the abnormally rapid destruction of an A-strain skin graft transplanted a few days later—the so-called " second set " response ; and (b) they excite the formation of humoral antibodies, among them haemagglutinins (Gorer, 1937), haemolysins (Hildemann, 1957) and cytotoxins (Gorer and O'Gorman, 1956). The antigens that provoke these 2 responses may or may not be the same, but because they have a common genetic determination they may well have similar immunologically determinant groups (Snell, 1957 ; Medawar, 1959*a*). If this is so, then antigens that sensitize their recipients to homografts should be capable of absorbing the corresponding humoral antibodies or of inhibiting their action. Thus an antigen prepared from the tissues of A-strain mice which excites transplantation immunity in CBA mice should absorb *in vitro* the haemagglutinins formed by the repeated injection of A-line lymphoid cells into CBA mice. Hildemann and Medawar (1959) found no convincing evidence that this was so, but their antigenic material—often in the form of nuclei or nuclear fragments—was particularly crude, and their failure to absorb humoral antibodies may have been due to the inaccessibility rather than to the absence of the appropriate combining sites. The evidence reported here shows that " transplantation antigens " extracted by the method of Billingham, Brent, and Medawar (1958) are indeed capable of absorbing or inhibiting the action of iso-antibodies causing the agglutination or lysis of A-strain red cells ; furthermore, their repeated injection into CBA mice provokes the formation of a high titre of haemagglutinins. This promises to provide a valuable auxiliary method of tracing antigenic activity through various preparative procedures, and ultimately of attempting a chemical identification.

MATERIALS AND METHODS

Mice.—We have used adult mice of strain A as donors, and of strains CBA or (less often) C3H as recipients.

Antigen was made from A-strain lymphoid tissue (spleen, lymph nodes, thymus) by the method of Billingham *et al.* (1958), modified in 2 minor ways : (a) at Stage 2 of their preparation a top-drive M.S.E. macerator was substituted for a blender of the piston and cylinder type to disperse the gelatinous cellular sediment in water : treatment for 2 min. sufficed, and the time of exposure to ultrasound was reduced from 60 to 30 sec. ; (b) at Stage 4, antigenic matter was spun down at 30,000 *g* instead of at 25,000 *g*. All absorption tests made use of the antigenic sediment produced by this final centrifugation, but the inhibition test made use of the " soluble " preparation (Stage 3) as it exists immediately prior to the final

centrifugation. Great pains were taken to standardize the preparation of antigen, particularly with respect to procedures affecting the degree of dispersion of the antigenic particles, and to make sure that the losses of substance at any stage were reduced to the very minimum. For want of a better method, one unit of antigenic matter is described as the quantity extracted from 1 mg. wet weight of lymphoid tissue. Four determinations gave 9·9, 9·2, 9·6 and 10·4 μg. as the dry weight of one unit of antigenic sediment. Antigenic preparations were tested for their power to sensitize *in vivo* by the method of Billingham *et al.* (1956, 1958).

Humoral antibodies were produced by the weekly intraperitoneal injection into CBA mice of the living cells (2–2·5 $\times$ 10^7 nucleated cells) expressed from 10 mg. wet weight of lymphoid tissue from CBA $\times$ A hybrid mice. The recipients were given 5 injections at weekly intervals, rested for 2 weeks, given a sixth injection, and bled from the heart a week later. Undiluted immune serum was stored in sealed ampoules at $-20°$. Antibodies were titrated as haemagglutinins by the method of Gorer and Mikulska (1954) or, for special purposes, as haemolysins by the method of Hildemann (1957), using absorbed rabbit complement (Owen, 1958). The 3 separate batches of antiserum used in the experiments described here gave titres as follows :—

serum batch	haemagglutinin titre	haemolysin titre
I	800–3200	400–800
II	3200	400–800
III	6400–12,800	1600–3200

Haemagglutination readings were taken by the slide method after 90 min. at 37° ; the degree of haemolysis was read after 60 min. and again after 120 min. at 37°. The haemagglutination-inhibition tests made use of a 3-part system : (i) 0·025 ml. antiserum in falling dilutions in 2 per cent dextran in 0·15 M NaCl ; (ii) 0·025 ml. of 0·15 M NaCl containing the substance suspected of inhibitory power ; and (iii) 0·025 ml. of a 3 per cent suspension of washed A-strain red cells in a heat-inactivated and absorbed human serum diluted with an equal volume of 0·15 M NaCl. Lysis inhibition tests made use of a 4-part system, with " Mg-saline " as diluent throughout (NaCl 0·14 M, sodium phosphate pH 7·6 0·01 M, $MgCl_2$ 0·001 M). The 4 components in order of addition were : (i) 0·05 ml. A-strain red cells washed twice in citrated saline and twice in Mg.-saline, and resuspended in the ratio 0·1 ml. whole blood to 5 ml. Mg.-saline ; (ii) 0·05 ml. of the substance suspected of inhibitory power ; (iii) 0·05 ml. antiserum in falling dilutions ; and, after a 5-min. interval, (iv) 0·05 ml. absorbed rabbit serum diluted by 1 volume to 4.

Absorptions were carried out by mixing various quantities of antigenic sediment redispersed in 0·15 M NaCl with equal volumes of 1/25 (batches I, II) or 1/50 (batch III) antiserum in 2 per cent dextran in 0·15 M NaCl. Absorption was allowed to proceed at room temperature for 20–25 min., and insoluble matter removed by centrifugation at 30,000 *g* for 30 min.

Most of our experiments on absorption or inhibition by antigenic extracts (as opposed to purified chemical substances) were founded on the haemagglutination test. The results of the lytic test were not at variance with those from the haemagglutination tests, but the strongly anticomplementary activity of the antigenic tissue extracts made the lytic technique unsuitable for routine use.

RESULTS

Specificity of absorption and inhibition.

By exposing 1·0 ml. 1/50 antisera I or II to varying quantities of antigenic sediment, it was found that 15 units of A-strain antigen would lower the haemagglutinin titre perceptibly and that 60 units removed all antibodies detectable within the chosen range of dilutions. Under exactly comparable conditions, 500–700 units of " antigen " made from the lymphoid tissues of CBA or C3H mice had no perceptible effect on the haemagglutinin titre. Representative titrations are shown in Table I. (Antiserum III was much more powerful : here, more than 400 units of A-strain antigen were required to exhaust the antibody present in

TABLE I.—*Haemagglutinin Titres After the Absorption, by Various Antigenic Extracts, of Antisera Prepared in CBA Mice Against A-strain Antigen*

Expt. No.	Serum batch	Source of antigen	Units of antigen per 1·0 ml. 1/50 antiserum	Antibody dilution 100	150	200	300	400	600	800	1200	1600	2400	3200	4800	6400	12,800
16	I	A	250, 125, 62	0		0		0		0		0		0			
			31	+		±		0		0		0		0			
			16	+++		++		+		±		0		0			
			0	++++		+++		++		±		0		0			
18	I	A	125, 62	0		0		0		0		0		0			
			31	±		±		0		0		0		0			
			16	+		±		±		0		0		0			
			8	++		++		+		±		0		0			
			0	++++		+++		+++		+		0		0			
2	I	CBA	765		+++		+++		+++		++		±		0		
		A	765		0		0		0		0		0		0		
			0		+++		+++		+++		++		+		0		
4	I	CBA	500		+++		+++		++		++		+		0		
		A	500		0		0		0		0		0		0		
			0		++++		++		++		++		+		0		
10	I	C3H	500		++++		++++		+++		+		±		0		
		A	500		0		0		0		0		0		0		
			0		++++		++++		+++		++		+		0		
46	III	A	800			0		0		0		0		0		0	0
			400			+		+		±		0		0		0	0
			200			++		+		±		0		0		0	0
			100			+++		++		+		±		0		0	0
			0			+++++		+++++		++++		+++		++		+	±

1·0 ml. 1/50 antiserum, though exposure to less than 100 units diminished the titre appreciably.)

The specificity of the action of A-strain antigen was made equally apparent by using the " soluble " antigenic preparation (*i.e.* the antigenic extract immediately before its final centrifugation : see above) as an inhibitor of agglutination. Adjusted to a concentration of 100 units per ml., A-strain antigen reduced the titre by not less than 4 halving dilutions (Tables II, III), but an exactly parallel preparation from CBA or C3H lymphoid tissues was quite inactive (Table II).

TABLE II.—*Use of " Soluble " Antigenic Extracts from A-strain, CBA, and C3H Mice to Inhibit the Agglutination of A-strain Red Cells by Antiserum I*

Expt. No.	Antigen concentration in units/ml.*	Source of antigen	Antibody dilution 150	300	600	1200	2400
1	135	A	+	±	0	0	0
	0		+++	+++	++	++	+
2	77	A	++	+	±	0	0
	77	CBA	+++	+++	+++	++	+
	0		+++	+++	+++	++	+
4	100	A	+	±	0	0	0
	100	CBA	++++	+++	+++	++	+
	0		++++	++	++	++	+
10	100	A	+	±	0	0	0
	100	C3H	++++	++++	+++	+	±
	0		++++	++++	+++	++	+

* The final concentration in the 3-part system is one third of the stated figure.

We took advantage of the specific inhibitory power of this " soluble " preparation to find out what proportion of antigenic activity during the preparation of the final sediment remains in the supernatant fluid after the final centrifugation at 30,000 *g* for 60 min. The four readings summarized in Table III show that

TABLE III.—*Inhibition-titration of A-strain " Soluble " Antigen Before and After Centrifugation at* 30,000 *g for* 60 *min., Using Antiserum I*

Expt. No.	Antigen concentration in units/ml.	Pre- or post-centrifugation	75	150	300	600	1200	2400
13	100	pre		0	0	0	0	0
	100	post		+	+	±	0	0
	0			+++	+++	++	++	±
14	100	pre		0	0	0	0	0
	100	post		++++	+++	++	±	0
	0			+++++	+++	++	+	±
16*	100	pre	0	0	0	0	0	0
	50	pre	+	0	0	0	0	0
	25	pre	++	±	0	0	0	0
	12½	pre	+++	++	±	0	0	0
	100	post	+++	++	±	0	0	0
	0		++++	+++	+	0	0	0
18*	100	pre	0	0	0	0	0	0
	50	pre	±	±	0	0	0	0
	25	pre	++	+++	++	+	0	0
	12½	pre	+++	++	++	+	±	0
	100	post	+++	+++	++	++	±	0
	0		+++	++++	+++	++	+	0

* The use of 1 per cent dextran is responsible for the low end-point titre in this experiment.

only about one-eighth of the activity remains in the supernatant fluid—a result which indicates that, at this stage of preparation, the power to absorb humoral antibody and to sensitize *in vivo* go hand in hand (Billingham *et al.*, 1958).

Direct effect of antiserum on the sensitizing power of antigen

The treatment of antigenic sediment with a great excess of specific antiserum *in vitro* had little if any effect upon its power to give rise to transplantation immunity *in vivo*. In each of 3 independent experiments, 1250 units of sedimented antigen were redispersed in 5·0 ml. freshly withdrawn and undiluted immune serum. After exposure for 30 min. the antigenic matter was recovered by centrifugation at 30,000 *g*, redispersed in Ringer-phosphate, and injected into 5 CBA mice at a dosage of 250 units per mouse. Five controls were treated in exactly the same way except for the replacement of antiserum by fresh normal serum from A-strain mice. All mice received test-grafts of A-strain skin 3 or 4 days after the injection of antigen, and the grafts were removed for histological examination 6 days later. The 2 sets of survival scores in the 3 experiments are recorded in Table IV : clearly there is no significant difference between them.

TABLE IV.—*Percentage of Epithelial Survival, at 6 days, in A-strain Skin Grafts on CBA Mice which had received 250 units of A-strain Antigen After its Exposure to Immune or to Normal Serum*

Expt No.	Titre of antiserum: Before absorption	Titre of antiserum: After absorption	Antigen treated with immune serum	Antigen treated with normal serum
403	1600	5120	25, 25, 5, 5, 5	50, 25, 25, 5, 5
405	1600	3200	100, 90, 75, 50, 5	50, 50, 25, 25, 5
415	6400	3200	50, 50, 5, 5, 5	50, 25, 25, 5, 5

These results are interesting because, as we shall record elsewhere—see Medawar (1959*b*) for a preliminary report—the injection of 1·0 ml. hyperimmune antiserum *in vivo* does indeed weaken the sensitivity produced by the injection of 250 units of antigen ; and the same is true when the injection of antigen precedes the injection of antiserum by as long as 3 days. Evidently this " enhancing" or " desensitizing " action of antiserum cannot be due to a simple direct inactivation of antigen by antibody. In the experiment referred to in Table IV the antigen : antibody absorption ratio was equivalent to only 5 units of antigen per 1·0 ml. 1/50 antiserum. It is not surprising, then, that the treatment did not lower the haemagglutinin titre of the antiserum ; on the contrary, the absorbed serum gave the higher titre in 2 experiments out of 3.

Production of antibodies by the injection of extracted antigens

Hildemann and Medawar (1959) were unable to satisfy themselves that the injection of isolated lymphoid nuclei or nuclear fragments could provoke the formation of humoral antibodies. Our present antigenic material can certainly do so. Five CBA mice received 250 units of A-strain antigen each by the intraperitoneal route on days 0, 5, 13, 18, 25 and 38, and were bled from the heart on the 45th day. Table V shows that haemagglutinins are formed in high titre. It also shows that the haemolytic titres fall far short of what would be expected of immunization by living lymphoid cells : the haemolytic titre of serum 2 (Table V)

TABLE V.—*Haemagglutinin and Haemolytic titres of the Sera of 5 CBA Mice which had received Six Injections of 250 units of Sedimented A-strain Antigen*

Serum No.	Agglutination endpoint	Haemolysis endpoint
1	2048	100
2	1024	<10
3	4096	200
4	2048	40
5	2048	50

was less than 10 where the agglutinin titre was about 1000, a fact which suggests that lysis and agglutination are not merely two different manifestations of the action of a single antibody.

A single intraperitoneal injection of 250 units of A-strain antigen into a CBA mouse is more than sufficient to bring about the total breakdown, within six days, of an A-strain skin graft transplanted 3 or 4 days later (Billingham *et al.*, 1958). Three of the 5 CBA mice hyperimmunized by extracted A-strain antigen were tested with A-strain skin 9 days after the last of their 6 injections of 250 units. Six days later the three grafts were found to carry some 50 per cent of surviving epithelium. The sensitivity of the recipients must therefore decline during the repeated administration of extracted antigen, just as it does during the repeated injection into CBA mice of living A-strain lymphoid cells (Medawar, 1959*b*). The very complex relationship between the survival time of a skin graft and the titre of humoral antibodies in its recipient will be discussed in another publication. For the present, it will be noticed that our results, so far as they go, do not uphold the tentative distinction between " T-antigens " and " H-antigens " in the terminology of Medawar (1959*a*) (see *Discussion*).

Stability of the antigenic sediment

Resistance to drying.—Drying in the frozen state does not abolish the absorptive power of the antigenic sediment. In spite of the fact that " lyophilized " tissues are regularly used to procure " enhancement "—*i.e.* to prolong the life of homografts by immunologically specific means—we now find that if the antigenic sediment is dried carefully its power to sensitize is not perceptibly impaired. In each of 5 independent trials, 1250 units of sedimented A-strain antigen were resuspended in 2–3 ml. water, shell-frozen at −79°, and dried for 3–4 hr. under vacuum (~0·001 mm. Hg) at a temperature not exceeding −5°. The material was reconstituted in Ringer-phosphate and injected into 5 CBA or C3H mice at a dosage of 250 units per mouse. Table VI summarizes the 6-day epithelial survival scores of A-strain skin grafts transplanted 3 days after the sensitizing injection. Their recipients were highly sensitive. In 5 further trials we found that drying could be continued overnight over phosphorus pentoxide under a vacuum of 0·01 mm. Hg without loss of sensitizing power, and that the dry preparation retained all or most of its activity after storage for more than 2 weeks under nitrogen at −20°. It follows that resistance to drying does not distinguish the power of an antigen to sensitize from its power to absorb humoral antibodies.

Effect of the periodate ion.—Kandutsch and Reinert-Wenck (1957) have shown that exposure to the periodate ion inactivates the antigens responsible for " enhancing " the growth of certain tumour homografts, and Billingham *et al.* (1958),

TABLE VI.—*Percentages of Surviving Epithelium, at 6 days, in A-strain Skin Homografts transplanted to CBA or C3H Mice Three Days after the injection of 250 units of Dried A-strain Antigenic Sediment*

(Expectation of survival of A-strain skin homografts 6 days after transplantation to normal CBA or C3H mice : 100.)

Expt. No.	"Lyophilized" antigen					Antigen further dried over P_2O_5					Notes
409	25,	5,	5,	0,	0						
411	25,	5,	5,	5,	0						
413	50,	0,	0,	0,	0						
416	50,	0,	0,	0,	0	0,	0,	0,	0,	0	
417	5,	0,	0,	0,	0	0,	0,	0,	0,	0	
420						0,	0,	0,	0		
423C						25,	25,	5,	0		Stored 7 days at 2°–3°.
423D						50,	5,	5,	5,	0	Stored 18 days at −20°.

found the same to be true of the sensitizing power of antigenic sediments. We have now re-investigated the matter, paying special attention to the possibility that products of the interaction between periodate and reducing agents might themselves have a damaging effect. Accordingly, 1250 units of A-strain antigen were exposed in the dark at 21° to 23° to sodium periodate in the presence of 0·1 M sodium phosphate pH 7·0 and 0·0375 M NaCl : the concentration of periodate was 0·005 M and its quantity (2 μM per 100 units of antigen). After 3 hr. 10 μM glycerol per 100 units of antigen were added to inactivate the periodate, and the antigenic matter was spun out at 30,000 *g* for 30 min. to eliminate the original reagents and their soluble reaction products. The sediment was resuspended in Ringer-phosphate and injected into CBA mice at a dosage of 250 units per mouse. A-strain skin grafts were transplanted 3 days after the injection of antigen and removed for examination 6 days later. Controls were of 2 kinds : those identical with the experimental series except that glycerol was added before instead of after the exposure to periodate, and those in which periodate was simply omitted from the reaction mixture. The results of 4 such experiments are summarized in Table VII, which reveals drastic impairment by active periodate but also some degree of impairment by inactivated periodate.

The power of periodate to weaken the absorptive power of sedimented antigen *in vitro* was investigated in much the same way. Antigenic sediment was suspended

TABLE VII.—*Percentages of Surviving Epithelium, at 6 days, in A-strain Skin Homografts Transplanted to CBA or C3H Mice 3 days after the injection of 250 units of Periodate-treated Antigen*

(See text for details of treatment and nature of controls.)

Expt. No.	Active periodate					Inactivated periodate					No periodate				
406	100,	100,	75,	50,	25*	50,	5,	5,	5,	0	0,	0,	0,	0,	0
408	100,	100,	100,	50,	25*	75,	75,	50,	50,	50	50,	25,	25,	5,	0
412	100,	100,	75,	75,	75	50,	25,	5,	5,	5	0,	0,	0,	0,	0
414	100,	100,	100,	75,	50	75,	5,	5,	5,	0	0,	0,	0,	0,	0

* Poorly-healed grafts, probably underscored.

in 0·005 M sodium periodate (8 μM per 100 units of antigen) in the presence of 0·05 M sodium phosphate pH 7·0 and 0·075 M NaCl (0·0375 M in exp. 40). After 19 hr. in the refrigerator the periodate was inactivated by the addition of 40 μM glycerol per 100 units of antigen. Antigen was recovered by centrifugation as before, and then used to absorb antiserum II in the ratio 1·0 ml. 1/50 antiserum to 31 units of antigen (50 units in expt 41). In the control experiments, glycerol was added before the experiment began instead of after it was over. The results of three such sets of experiments, summarized in Table VIII, show that the titres of antibody exposed to a periodate-treated antigen are very little inferior to the titres of normal antibody.

TABLE VIII.—*Haemagglutinin Titres of Antiserum II after Absorption with Periodate Treated Antigen*

(For details of exposure and nature of controls, see text.)

Expt. No.	Nature of treatment	Antibody dilution 50	100	200	400	800	1600	3200
39	Active periodate	+++	+++	++	+	±	0	0
	Inactivated periodate	0	0	0	0	0	0	0
	No periodate	0	0	0	0	0	0	0
	Normal antibody	++++	++++	+++	+++	++	+	±
40	Active periodate		++++	+++	++	+	±	0
	Inactivated periodate		++	+	0	0	0	0
	Normal antibody		++++	++++	+++	++	+	±
41	Active periodate	++++	+++	+++	++	++	+	0
	Inactivated periodate	0	0	0	0	0	0	0
	Normal antibody	++++	++++	++++	+++	++	+	0

Effect of formaldehyde.—A suspension of 1250 units of A-strain antigen was exposed for 19 hr. at 2–3° to 4·0 ml. 0·1 M formaldehyde in 0·01 M sodium phosphate at pH 7·0. The antigen was recovered by centrifugation and injected into 5 C3H mice at a dosage of 250 units per mouse. This treatment had little if any effect on the sensitizing power of the antigen, for the 6-day epithelial survival scores of A-strain test grafts transplanted 3 days after the injection were, in 2 experiments, 5, 5, 5, 5, 0 and 25, 0, 0, 0, 0, respectively. The effect of such a treatment upon the power of A-strain antigen to absorb antiserum III is summarized in Table IX : something between 25 and 50 per cent of the original activity remained.

Effect of heat.—The exposure of a suspension of A-strain antigen in water to a temperature of 57° for 20 min. reduced its absorptive power to between 25 and 50 per cent of its original value. A number of not very systematic tests on antigenic preparations of various kinds showed that this degree of exposure to heat also weakened the power to sensitize *in vivo*, though without abolishing it completely. We did not pursue these experiments, because their interpretation is ambiguous : inactivation might be due to a very rapid enzymatic destruction in the temperature zone between 40°–50° during warming. On the other hand they led us to expect that heating to 100° for 4 min. would inactivate the antigen completely. So far as concerns its absorptive power, this expectation was fulfilled : Table X shows that antigen heated to 100° for 4 min. retained no trace whatsoever of its absorptive power. Most unexpectedly, however, 6 independent trials

TABLE IX.—*Haemagglutinating Activity of Antiserum III after Absorption with A-strain Antigen Exposed to M/10 Formaldehyde*

(For details of exposure, see text.)

Expt. No.	Treatment of antigen	Units of antigen per 1·0 ml. 1 : 50 antiserum	Antibody dilution 200	400	800	1600	3200	6400	12,800
45	Formaldehyde	400	+++	++	+	±	0	0	0
	Untreated	75	++++	++++	+++	++	+	±	0
	Unabsorbed	0	++++	++++	++++	+++	+++	+	0
46	Formaldehyde	400	++	+	+	0	0	0	0
	Untreated	800	0	0	0	0	0	0	0
	,,	400	+	+	±	0	0	0	0
	,,	200	++	+	±	0	0	0	0
	,,	100	+++	++	+	±	0	0	0
	Unabsorbed	0	+++++	+++++	++++	+++	++	+	±
50	Formaldehyde	500	++	+	+	0	0	0	0
	Untreated	500	+	0	0	0	0	0	0
	Unabsorbed	0	++++	++++	+++++	+++	++	+	±

TABLE X.—*Haemagglutinating Activity of Antisera absorbed with A-strain Antigen heated to 100° for 4 min.*

Expt. No.	Anti-serum	Treatment of antigen	Units antigen per 1·0 ml. 1 : 50 antiserum	Antibody dilution 150	300	600	1200	2400	
10	I	100°	500	++++	++++	+++	++	+	
		Nil	500	0	0	0	0	0	
		Unabs.	0	++++	++++	+++	++	+	
				200	400	800	1600	3200	6400
40	II	100°	125	++++	+++	+++	++	0	0
		Nil	31	++	0	0	0	0	0
		Unabs.	0	++++	+++	++	+	±	0
64	III	100°	1000	++++	+++	+++	++	++	+
		Unabs.	0	+++	++++	+++	++	++	+

have shown that antigen heated in aqueous suspension to 100° for 4 min. or even for 12 min. retains a small but clearly discernible fraction of its power to sensitize *in vivo*. These experiments are still in progress : the heated antigen might conceivably act through a general non-specific activation of its recipients' immunological response, a possibility that is now under investigation.

Effect of ethanol.—The effect of 75 per cent v/v aqueous ethanol upon A-strain antigen is to abolish its absorptive power or to reduce it to a level that cannot exceed one-eighth of its original value. This generalization applied with equal force to preparations of three kinds : (a) the precipitate formed almost instantly at 0° by the action of 75 per cent ethanol on a suspension of antigen in 0·05 M sodium acetate at pH 7·0 ; (b) the lipid-containing matter extracted into 75 per cent ethanol under the same conditions ; and (c) an unfractionated preparation. Ethanol was removed from preparations of types (b) and (c) either on a rotary evaporator below room temperature or by dialysis against distilled water under

TABLE XI.—*Effect of 75 per cent v/v Ethanol on the Absorptive Power of A-strain Antigen*

Expt. No.	Anti-serum	Treatment of antigen	Units antigen per 1·0 ml. 1/50 antiserum	Antibody dilution 100	200	400	800	1600	3200	6400	12,800
21	I	Ethanol S	125	+++	++	+	0	0	0		
		Untreated	31	+	±	±	0	0	0		
		Unabsorbed	0	++++	++++	++	++	+	±		
22	II	Ethanol ER	300	++++	++++	+++	++	+	0		
		Ethanol ED	500	++++	++++	+++	++	0	0		
		Unabsorbed	0	++++	++++	+++	++	+	0		
39	II	Ethanol ER*	250	++	+++	±	0	0	0		
		Untreated	31	0	0	0	0	0	0		
		Unabsorbed	0	++++	+++	+++	++	+	±		
44	III	Ethanol TD	100	+++++	+++++	++++	+++	++	+	0	
		Untreated	31	++++	+++	+++	++	+	0	0	
		Unabsorbed	0	+++++	+++++	++++	++++	+++	+	±	
50	III	Ethanol TD	500		+++++	+++++	++++	+++	++	+	0
		Ethanol TR	500		+++++	+++++	++++	+++	++	+	±
		Untreated	500		+	0	0	0	0	0	0
		Unabsorbed	0		++++	++++	+++++	+++	++	+	±

S = Sediment precipitated by ethanol in the presence of acetate ions. E = Matter extracted into 75 per cent ethanol and not deposited by centrifugation at 5000 **g**. T = Unfractionated total preparation after exposure to ethanol. R = Ethanol removed by evaporation. D = Ethanol removed by dialysis.

* Extraction in aqueous ethanol: with no acetate to promote flocculation, the extract here contains matter that would have appeared in the sediment in Expt. 21.

nitrogen at a pressure of about 30 cm. Hg. The more informative experiments are summarized in Table XI.

Experiments too detailed to be summarized here show that preparations of type (a)—the precipitates formed instantly at 0° by adding 3 vols ethanol to 1 vol. antigen resuspended in 0·03 M to 0·05 M neutral sodium acetate or in 1·0 M $MgCl_2$—retained no more than a just perceptible fraction of their power to sensitize *in vivo*. (The mere exposure of a compact antigenic sediment to 75 per cent ethanol by diffusion at 2°–3° overnight was equally damaging.) Preparations of type (b), representing the matter extracted into 75 per cent ethanol, were quite inactive; and those "total" preparations of type (c) from which ethanol was removed by evaporation had no greater activity than preparations of type (a). However, preparations of type (c) from which ethanol had been removed by dialysis against distilled water overnight regained a high proportion of their sensitizing power—a property that distinguishes the absorptive from the sensitizing activities of the antigenic preparation, though the distinction may be trivial (see *Discussion*).

Inhibition Tests with Purified Substances

We have investigated the power of a number of oligo- and polysaccharides to inhibit the agglutination or lysis of A-strain red cells (*cf.* Albert and Lejeune-Ledant, 1959). Unhappily, most of the polysaccharides and some of the oligosaccharides promote agglutination non-specifically—a property presumably akin to that for which dextran itself was chosen as an adjuvant in the haemagglutination technique of Gorer and Mikulska (1954)—and other substances, like human blood-group A or B substance, are disqualified from the agglutination test because (see *Materials and Methods*) it makes use of human antibody-containing serum. Most of our tests are therefore of necessity based upon the inhibition of lysis.

Table XII is a list of the substances tested. With the following exceptions, all were inactive. A feeble inhibition of lysis (a reduction of the apparent titre to about one quarter, *i.e.* by 2 or 3 tubes) was given by 2 per cent solutions of 3 different samples of purified human blood-group A substance (though not by B, H, or Le^a substances); by Type XIV pneumococcal polysaccharide (though not by Types I, II, and V); and by a 2 per cent solution of a *Shigella shigae* polysaccharide. Rather stronger inhibition (with one sample, >4 and >5 tubes) was given by 2 different samples of purified hog gastric mucin with A but no H activity, whereas hog gastric mucins with H but very little A activity were quite ineffective. Thus the actively inhibitory substances were those distinguished by "Forssman" affinities. The degree of inhibition was unaffected by the addition of further complement. In view of the fact that human A-substance was active where such a close chemical relative as human B-substance was not, it seems possible, provisionally at least, to take these results at their face value. It may be added that the one sample we had of human M substance was also weakly inhibitory, but as human MN-substance was not we cannot at present build anything upon this observation.

DISCUSSION

The experiments described above show that, if the strains of donors and recipients are judiciously chosen, conventional serological methods can be used to study the physical and chemical properties of the iso-antigens associated with

TABLE XII.—*List of Purified or Chemically Defined Substances that have been Examined for their Power to Inhibit the Agglutination or Lysis of A-strain red Cells by Specific Antisera.*

A = Tested for agglutination-inhibition.
L = Tested for inhibition of lysis.

The final concentrations of the substances tested were one-third of the stated values in the agglutination tests and one-fourth of the stated values in the lytic tests.

A	D-Ribose 2 per cent.
A	2-Deoxy-D-ribose 2 per cent.
AL	D-Mannose 2 per cent.
AL	D-Galactose 2 per cent.
A	D-Glucose 2 per cent.
A	L-Fucose 2 per cent.
A	L-Rhamnose 2 per cent.
AL	α-Methyl-galactoside 2 per cent.
AL	β-Methyl-galactoside 2 per cent.
AL	α-Methyl-mannoside 2 per cent.
AL	Lactose 2 per cent.
AL	Raffinose 2 per cent.
AL	Fucosido-lactose 2 per cent.
L	Lacto-N-tetraose 1 per cent.
L	Lacto-difucotetraose 1 per cent.
L	Lacto-N-fucopentaose I 1 per cent.
L	Lacto-N-fucopentaose II 1 per cent.
L	Lacto-N-difucohexaose 1 per cent.
L	Lactosamine 1 per cent.
AL	N-Acetyl-D-glucosamine 2 per cent.
AL	N-Acetyl-D-galactosamine 2 per cent.
AL	Glucuronic acid, Na salt, 1 per cent.
AL	N-Glycolylneuraminic acid, Na salt, 1 per cent.
AL	N-Acetylneuraminic acid, Na salt, 1 per cent.
AL	O, N-Diacetylneuraminic acid, Na salt, 1 per cent.
L	Human Blood Group Substances A*, B, H, Le^a 2 per cent ; M, N, MN 1 per cent.
L	Hog gastric mucin A-active*, H-active, 0·2–2 per cent.
L	Pneumococcal polysaccharides types I, II, V, XIV*, 1 per cent.
L	Bacterial polysaccharides *B. anthracis, B. megatherium, Past. pestis, Sh. shigae** 1–2 per cent.
L	Dextran 2 per cent.
L	Sialomucopolysaccharide 0·2 per cent.

* Substances with some activity : see text.

transplantation immunity in mice. Although strains A and CBA (or C3H) differ at many genetic loci controlling differences between the survival times of homografts (Barnes and Krohn, 1957), the activity of A-strain antigen *in vivo* and *in vitro* is dominated by " strong " antigenic differences at the Histocompatibility-2 locus (see Gorer, 1956, and Berrian and Jacobs, 1959 : strain A has the composition H-2*a* and strains CBA and C3H the composition H-2*k*).

The absorption and inhibition tests bear directly on the properties of A-strain antigens that are associated with the formation of humoral antibodies. To what extent are they relevant to their sensitizing activity, *i.e.* to the fact that, after injection into mice of strains CBA or C3H, they curtail the life of A-strain skin grafts transplanted a few days later ? All that can be said so far is that the two activities go hand in hand during the crude extractions so far attempted, and that, with two exceptions, physical or chemical treatments that reduce or abolish the

one activity will reduce or abolish the other. These results do not therefore uphold the distinction (Medawar, 1959) between " T-antigens " that sensitize and " H-antigens " that provoke the formation of humoral antibodies or inhibit their action *in vitro*. Drying and treatment by formaldehyde were equally innocuous and treatment by periodate or by ethanol were equally damaging to both activities. The two exceptions were these : (a) heating to 100° abolished the power to absorb antibody, but left a well-defined residue of sensitizing power ; and (b) it was possible to recover the sensitizing but not the absorptive activity by the prolonged dialysis of preparations exposed to 75 per cent ethanol. The differences, though genuine, may be unimportant : with such crude preparations, the effect of " fixation " by ethanol or heat may simply render combining sites inaccessible to antibody *in vitro* ; alternatively the heat-stable antigen may belong to a different antigenic system.

At present it is hardly possible to interpret the physical and chemical properties we have described in terms of chemical constitution. The clue offered by the inhibitory action of polysaccharides with Forssman affinities is a very tenuous one, but it points in the same direction as evidence from other sources (Kandutsch and Reinert-Wenck, 1957 ; Billingham *et al.*, 1958), viz : towards the complicity of carbohydrate substances in the immunologically specific reactions of the antigens associated with transplantation immunity in mice.

SUMMARY

Extracted cell-free antigens of A-strain origin that can sensitize CBA or C3H mice to homografts can also (a) excite the formation of humoral antibodies, and (b) specifically inhibit the agglutination or lysis of A-strain red cells by humoral antibodies *in vitro*. Their methods of preparation and their resistance to various physical and chemical treatments (drying, heating to 100°, exposure to periodate, ethanol or formaldehyde) do not justify drawing a distinction between sensitizing antigens and antigens that excite the formation of humoral antibodies. Polysaccharides with Forssman affinities have some discernible power to inhibit the lysis of A-strain red cells by specific antisera.

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REFERENCES

ALBERT, F. AND LEJEUNE-LEDANT, G.—(1959) In ' Biological Problems of Grafting '. Oxford (Blackwell), p. 109.

BARNES, A. D. AND KROHN, P. L.—(1957) *Proc. roy. Soc. B*, **146**, 505.

BERRIAN, J. H. AND JACOBS, R. L.—(1959) In ' Biological Problems of Grafting '. Oxford (Blackwell), p. 131.

BILLINGHAM, R. E., BRENT, L. AND MEDAWAR, P. B.—(1956) *Nature, Lond.*, **178**, 514.
—(1958) *Transplant. Bull.*, **5**, 377.
GORER, P. A.—(1937) *J. Path. Bact.*, **44**, 691.—(1956) *Advanc. Cancer Res.*, **4**, 149.
Idem AND MIKULSKA, Z. B.—(1954) *Cancer Res.*, **14**, 651.
Idem AND O'GORMAN, P.—(1956) *Transplant. Bull.*, **3**, 142.
HILDEMANN, W. H.—(1957) *Ibid.*, **4**, 148.
Idem AND MEDAWAR, P. B.—(1959) *Immunology*, **2**, 44.
KANDUTSCH, A. A. AND REINERT-WENCK, U.—(1957) *J. exp. Med.*, **105**, 125.
MEDAWAR, P. B.—(1959*a*) In ' Biological Problems of Grafting '. Oxford (Blackwell), p. 6.—(1959*b*) *Ibid.*, p. 19.
OWEN, R. D.—(1958) *Radiation Res.*, **9**, 164.
SNELL, G. D.—(1957) *Annu. Rev. Microbiol.*, **11**, 439.